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Title: Formation of a single polar flagellum by two distinct flagellar gene sets in *Sphingomonas* sp. strain A1

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Abbreviations: Strain A1, *Sphingomonas* sp. strain A1; TEM, transmission electron microscopy; CBB, Coomassie Brilliant Blue R-250

Footnote: The nucleotide sequences of strain A1 flagellar and chemotaxis genes in this paper were deposited in the GenBank/EMBL/DDBJ databases under the following accession numbers: *sph1784-1824*, LC043068; *sph183-184*, LC043069; *sph223-224*, LC043070; *sph241-245*, LC043071; *sph539-544*, LC043072; *sph1045-1058*, LC043073; *sph3884-3889*, LC043074; *sph3931-3933*, LC043075, *sph3939-3951*, LC043076; *p5'*, AB968520; *sph183*, LC016694; *sph184*, LC016695; *sph1784*, LC016662; *sph1786*, LC016663; *sph1788*, LC016664; *sph1789*, LC016665; *sph1790*, LC016666; *sph1791*, LC016667; *sph1792*, LC016668; *sph1793*, LC016669; *sph1794*, LC016670; *sph1795*, LC016671; *sph1796*, LC016672; *sph1798*, LC017868; *sph1800*, LC016674; *sph1801*, LC016675; *sph1802*, LC016676; *sph1803*, LC016677; *sph1804*, LC016678; *sph1807*, LC016679; *sph1808*, LC016680; *sph1809*, LC016681; *sph1810*, LC016682; *sph1811*, LC016683; *sph1812*, LC016684; *sph1813*, LC016685; *sph1814*, LC016686; *sph1816*, LC016687; *sph1817*, LC016688; *sph1818*, LC016689; *sph1819*, LC016690; *sph1820*, LC016691; *sph1821*, LC016692; *sph1822*, LC016693; *sph241*, LC016780; *sph242*, LC016781; *sph243*, LC016782; *sph244*, LC016783; *sph245*, LC016784; *sph539*, LC016785; *sph540*, LC016786; *sph541*, LC016787; *sph542*, LC016788; *sph543*, LC016789; *sph544*, LC016790; *sph1046*, LC016791; *sph1047*, LC016792; *sph1048*, LC016793; *sph1049*, LC016794; *sph1050*, LC016795; *sph1051*, LC016796; *sph1052*, LC016797; *sph1053*, LC016798; *sph1054*, LC016799; *sph1057*, LC016800; *sph1058*, LC016801; *sph3884*, LC016802; *sph3885*, LC016803; *sph3886*, LC016804; *sph3887*, LC017869; *sph3888*, LC016805; *sph3889*, LC016806; *sph3931*, LC016807; *sph3932*, LC016808; *sph3933*, LC016809; *sph3939*, LC016810; *sph3940*, LC016811; *sph3941*, LC016812; *sph3942*, LC016813; *sph3944*, LC016814; *sph3945*,

LC016815; *sph3946*, LC016816; *sph3947*, LC016817; *sph3948*, LC016818; *sph3951*, LC016819; *sph821*, LC016820; *sph1226*, LC016821; *sph1229*, LC016822; *sph1228*, LC016823; *sph3661*, LC016824; *sph1225*, LC016825; *sph2312*, LC016826; *sph3660*, LC016827; *sph3662*, LC016828; *sph3741*, LC016829; *sph3649*, LC016830; *sph2313*, LC016831; *sph1230*, LC016832; *sph3659*, LC016833; *sph1231*, LC016834; *sph3658*, LC016835; *sph2859*, LC016836; *sph2942*, LC016837; *sph2504*, LC016874; *sph1232*, LC017709.

A Gram-negative *Sphingomonas* sp. stain A1, originally identified as a non-motile and aflagellate bacterium, possesses two sets of genes required for flagellar formation. In this study, we characterised the flagellar genes and flagellum formation in strain A1. Flagellar gene cluster set I contained 35 flagellar genes, including one flagellin gene (*p6*), where the gene assembly structure resembled that required for the formation of lateral flagella in gamma-proteobacteria. The set II flagellar genes were arranged in eight shorter clusters with 46 flagellar genes, including two flagellin genes (*p5* and *p5′*) and *flhF*, which is required for polar flagella. Our molecular phylogenetic analysis of the bacterial flagellins also demonstrated that, in contrast to p5 and p5′, p6 was categorised as a lateral flagellin group. The motile phenotype appeared in strain A1 cells when they were sub-cultured on semisolid media. The motile strain A1 cells produced a single flagellum at the cell pole. DNA microarray analyses using non-motile and motile strain A1 cells indicated that flagellar formation was accompanied by increased transcription of both flagellar gene sets. The two flagellins p5 and p6 were major components of the flagellar filaments isolated from motile strain A1 cells, indicating that the polar flagellum is formed by lateral and non-lateral flagellins.

INTRODUCTION

Flagella are structures used for cell locomotion by various bacteria and there are several types, which depend on the number and position of the flagella. In general, flagella are classified into four types according to the flagellar position on the bacterial cell, i.e. peritrichous, polar, sub-polar and lateral flagella (Leifson, 1960). In general, one flagellar system is present but some bacteria, including *Aeromonas*, *Azospirillum*, *Rhodospirillum* and *Vibrio* species, possess two distinct flagellar systems (McCater, 2006; Merino *et al*., 2006). These bacteria constitutively express a polar flagellum via one flagellar system and environmentally-induced lateral flagella via the other system. Although the flagella produced by the one flagellar system often affect the transcription of genes required for the formation of the other flagella (Merino *et al*., 2006), distinct flagellar systems are regulated primarily by different factors and they form flagella at different positions.

An individual flagellum comprises three major sub-structures: the filament, hook and basal body. The assembly of these sub-structures is controlled by a hierarchy that governs the transcription of flagellar genes. The regulatory hierarchies of flagellar gene expression have been analysed comprehensively in some bacterial flagella, such as the peritrichous flagella of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Chilcott & Hughes, 2000), polar flagella in *Pseudomonas aeruginosa* (Dasgupta *et al*., 2003) and the polar and lateral flagella in *Vibrio parahaemolyticus* (Stewart & McCarter, 2003; Kim & McCarter, 2000) and *Aeromonas hydrophila* (Wilhelms *et al*, 2013; Canals *et al*., 2006). The regulatory mechanism is different in each of these bacteria. For example, the flagellar genes are divided into three classes in *S. enterica*

(classes 1 to 3). The master gene operon *flhDC* in class 1 activates class 2 operons, which contain genes for the hook and basal body, with the help of a typical sigma factor σ70. A flagellar-specific sigma factor (σ28) called FliA is expressed from the class 2 operons, which then activates the class 3 operons to facilitate filament formation and chemotaxis. In *P. aeruginosa*, the flagellar genes are classified into four classes, which are governed by σ54. The two distinct flagellar systems in *Aeromonas* and *Vibrio* species are regulated separately by different transcriptional hierarchies.

Sphingomonas sp. strain A1 (strain A1), one of the alpha-proteobacteria, is a Gram-negative bacterium, which was originally isolated from soil as an alginate-assimilating microbe (Yonemoto *et al*., 1991). Strain A1 forms characteristic 'pits' on the cell surface to incorporate alginate, depending on the presence of extracellular alginate. Strain A1 has been identified as a non-motile and aflagellate bacterium, although flagellin homologous proteins (p5 and p6) are expressed on the bacterial cell surface, one of which possesses a potent alginate-binding ability (Hashimoto *et al*., 2005). In the present study, we characterised flagellar formation in strain A1 through genome-based gene analysis, DNA microarrays and electron microscopy. The components of the strain A1 flagellar filaments were also analysed, which showed that the two distinct types of flagellins form a single polar flagellum.

METHODS

Bacterial strains and culture conditions. Non-motile wild-type and motile (A1-M5) strain A1 cells were used in this study. Bacterial cells were cultured aerobically at 30°C in ALY medium [0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.01%

118 $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract and 0.5% sodium alginate; pH 7.2] or AHY medium
119 [0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% Na_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.5% yeast
120 extract and 0.5% sodium alginate; pH 7.2]. For plate culture experiments, agar was
121 included in the AHY medium at a final concentration of 0.5 or 0.3%. For molecular
122 identification of each flagellin (p5, p5', or p6) in the filament fraction, *p5* and *p6*
123 gene-disruptants (Hashimoto *et al*., 2005) were grown in the ALY medium. In the
124 genome of these gene-disruptants, kanamycin resistant gene is inserted into the center
125 region of the corresponding gene.
126
127 **Isolation of motile strain.** The motile cells were obtained using semisolid AHY plates
128 with 0.5% agar as described previously (Negrete-Abascal *et al*., 2003). A single colony
129 of strain A1 (each non-motile strain of wild-type, *p5* gene-disruptant and *p6*
130 gene-disruptant) grown on ALY plate solidified with 1.5% agar was sub-cultured on
131 semisolid AHY plates and incubated at 30°C. The cells at the edge of a single colony
132 formed were sub-cultured to a fresh semisolid AHY plate and were incubated at 30°C.
133 In each experiment, cells of the motile strain were picked from the edge of colony
134 formed on semisolid AHY plate which was sub-cultured periodically.
135
136 **DNA microarray.** Total RNA was extracted using the hot phenol method from
137 wild-type strain A1 and A1-M5 cells grown in ALY medium to an OD_{600} of 0.5 to 0.6 as
138 described previously (Hayashi *et al*., 2014). The resultant RNA was hybridized to strain
139 A1 DNA microarray chip designed by Roche NimbleGen. The gene chip contains 3985
140 strain A1 genes fixed on a glass slide obtained by fixing two sets of 9 unique probes

141 composed of 60-mer synthetic oligonucleotides for each gene. Labeling with cyanine
142 (Cy3), fragmentation, and hybridization were carried out by Roche NimbleGen in line
143 with NimbleGen Array User's Guide: Gene Expression Arrays Version 5.1 (Roche
144 NimbleGen, 2010). Arrays were scanned at 532 nm and a resolution of 2 µm with a
145 NimbleGen MS200 microarray scanner and analyzed by quantile normalization and
146 robust multiarray averaging (Irizarry *et al*., 2003a; Irizarry *et al*., 2003b). The resultant
147 about 18 raw expression data for each gene were subjected to statistical treatment. The
148 normalized data were processed by a program of the NANDEMO Analysis 1.0.0 (Roche
149 diagnostics). Student's t-test was adopted for analyzing the mean log ratios of two
150 samples and subsequent Bonferroni adjustment for multiple testing (3985 genes on
151 arrays) was used as a rigorous criterion for significant changes in signal intensity.
152 Changes with $P < 0.05$ were considered statistically significant. The microarray data
153 obtained in this study have been deposited in the Gene Expression Omnibus database at
154 NCBI under accession number GSE62901.
155
156 **Microscopy.** Light microscopy was performed using an Olympus BX51 microscope.
157 A1-M5 cells were grown overnight aerobically at 30°C in ALY medium. Transmission
158 electron microscopy (TEM) (JEOL JEM-1200EX) was performed after negative
159 staining with 2% phosphotungstic acid in a similar manner to that described previously
160 (Hisano *et al*., 1995).
161
162 **Isolation of flagella.** Flagellar filaments were isolated from the cells, as described
163 previously (Iida *et al*., 2009) but with some modifications. Briefly, A1-M5 cells were

cultured aerobically in AHY medium (10 mL) to an OD_{600} of ca 1.0 and harvested by centrifugation at 4°C (6,000 × *g* for 10 min). The pellet was resuspended in 1 mL of 10 mM Tris-HCl (pH 7.5) and passed through a needle (22 gauge) 10 times to shear off the flagella from the cells. After removing the cells by centrifugation (10,000 × *g* for 10 min) at 4°C, the flagella were pelleted by ultracentrifugation (100,000 × *g* for 60 min) at 4°C and resuspended in 50 µL of 10 mM Tris-HCl (pH 7.5).

Western blotting. Bacterial cells grown in ALY or AHY medium at 30°C were harvested by centrifugation (6,000 × *g* for 10 min) at 4°C, resuspended in 20 mM Tris-HCl (pH 7.5) and lysed with SDS. The cell lysates or isolated flagellar filaments were subjected to SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore) at 1.5 mA/cm^2 for 30 min. The immunoreactions were performed according to the rapid immunodetection method described in the manufacturer's instructions (Millipore) using rabbit anti-p5 antisera (Hashimoto *et al*., 2005) or rat anti-p6 antisera (Kobayashi, Graduation Thesis of Kyoto University, pp9-10, 2013) as a primary antibody and donkey anti-rabbit or goat anti-rat immunoglobulin antibody (GE Healthcare) as a secondary antibody. Both antibodies to p5 and p6 purified from recombinant *E. coli* cells were raised in rabbit and rat, respectively. The resultant sera were used as polyclonal antibodies. Immunoreactive proteins were detected using a POD immunostain set (Wako) or EzWestLumiOne (ATTO).

Gene analyses. The MEME program (Bailey & Elkan, 1994) was used to extract the common motif from the upstream sequences of the flagellar genes. The primary

sequence identities of the flagellins were calculated using the BLAST program (Madden *et al.*, 1996). A molecular phylogenetic tree was constructed using CLUSTALW (Thompson *et al.*, 1994) and the TreeView program (Page, 1996).

RESULTS AND DISCUSSION

Two sets of flagellar genes in strain A1

The complete genome sequence of strain A1 was determined previously and the individual genes have been annotated, although the genome sequence data has not yet been published. The genome size of strain A1 is approximately 4.6 Mb with about 4000 genes. Strain A1 has been assigned as a non-motile and aflagellate bacterium (Yonemoto *et al.*, 1991), but we found that its genome contains at least 80 flagellar genes (Fig. 1). A large gene cluster from *sph1784* to *sph1824* (Fig. 1(a)) appeared to contain almost all of the genes required for flagellum formation. Other smaller clusters that contained 2–12 flagellar genes were also found in the genome (Fig. 1(b)). Most of the genes in the smaller clusters overlapped with the genes in the larger cluster and thus these genes alone appeared to be sufficient for flagellar formation. Therefore, we designated the larger cluster of flagellar genes as set I and the shorter clusters as set II.

The flagellar gene arrangement is known to differ among species, but the arrangement of lateral flagellar genes is somewhat conserved in gamma-proteobacteria (Merino *et al.*, 2006; Ren *et al.*, 2005). The arrangement of strain A1 gene cluster set I was similar to that of the lateral flagellar genes. *lafK* encodes a regulatory protein for lateral flagellar gene expression (Stewart & McCarter, 2003) and it is usually included in the *fliEFGHIJ* operon, but it was not present in the set I cluster (Fig. 1(b)).

Most of the genes required for flagellar formation (Aizawa, 2014) were found in both the set I and II clusters. In addition to the essential genes required for flagellar production, the set II clusters contained *flhC* and *flhD*, which are present in the genomes of beta- and gamma-proteobacteria such as *Salmonella* and *Escherichia*, although strain A1 is a member of alpha-proteobacteria. Heterotetramer of FlhD2C2 is known to regulate the transcription of other flagellar gene operons, typically *fliAZ*, *flhBA*, *flgAMN* and *flgBCD* (Claret & Hughes, 2002). The sequence identities between strain A1 *flhC* and *flhD* genes and thieir corresponding genes of *E. coli* are 54 and 31%, respectively. We searched the common motives in the upstream sequences of the strain A1 *fliA*, *flhB*, *flgA* and *flgB* genes, but failed. Instead, the homologous sequence (5′-AAGCGGCCGAATAGCCggggcttttcCCGCATTTATCCGGGC-3′) to FlhD2C2-binding motif (5′-AATGGCAGAAATAGCG $N_{10\text{-}12}$ CGCTATTTCTGCCATT-3′) (Stafford *et al*., 2005) was found only in the upstream sequence of the set II *flhB* (*sph3884*) gene. The *flhF* and *flhG* genes are involved in the orientation of the flagella at the cell pole and regulation of the number of flagella, respectively, and they are present in the genomes of bacteria with a single polar flagellum (Kazmierczak & Hendrixson, 2013, Kusumoto *et al*., 2006). In strain A1, *flhF* and *flhG* were present in the set II genes, thereby suggesting that the set II flagellar genes are involved in the formation of a single polar flagellum.

Two flagellin homologues, p5 and p6, were identified previously in the outer membrane fraction of strain A1 (Hashimoto *et al*., 2005). We found that another flagellin gene, *p5′*, was also encoded in the downstream region of *p5* (Fig. 1(b)). The shared amino acid sequence identities between p5′ and p5 and between p5′ and p6 were

85% and 40%, respectively. The amino acid length of p5′ (384 residues) was also similar to that of p5 (383 residues), but different from that of p6 (297 residues). Although *p5* and *p5′* are neighbouring, a possible terminator sequence (5′-AACAAAAAACCCGCCTTGTGCGGGTTTTTTGTT-3′) was inserted between them, thereby suggesting that there was no operon structure in *p5* and *p5′*. In fact, the sequence 5′-TAAGTTT X_{11} GCCGATA-3′ (Ide *et al*., 1999), which is recognised specifically by sigma factor σ28 during flagellar formation, was located in the region upstream of the start codon of *p5* (5′-TTCAAGTTTcaaagcgccggGCCGATG-3′), but not in that of *p5′*. Instead, the sequence 5′-CTGGCCcgccctTTGCA-3′ located upstream of *p5′* is probably recognised by σ54 (Barrios *et al*., 1999). The consensus sequences for σ28 recognition were situated in other sites in the strain A1 flagellar genes. All of the σ28-related genes (*sph223*, *sph242*, *sph246*, *sph537* and *sph1057*) shown in Table 1 were included in set II, and none were found that belonged to set I.

Characterisation of strain A1 flagellin genes

A phylogenetic tree was constructed using flagellins from bacteria with two flagellar systems and from sphingomonads with analysed genomes (Fig. 2). Most of the bacteria with two flagellar systems constitutively produce a mono-polar flagellum and lateral flagella are induced by change in environmental conditions, such as viscosity, pressure and aeration (McCarter, 2004; Eloe *et al*., 2008; Shimada *et al*., 1985; Moens *et al*., 1995; Sachett *et al*., 1997), except in the following three bacterial strains: *E. coli* strain 042 forms peritrichous flagella without expressing a second flagellar gene system (Ren *et al*., 2005); *Rhodobacter sphaeroides* constitutively forms a single sub-polar flagellum,

256 but it produces a polar flagellum in aerobic growth conditions (Poggio *et al*., 2007);
257 *Bradyrhizobium japonicum* forms a single thick flagellum and multiple thin flagella in a
258 sub-polar position, which are sometimes called lateral flagella (Kanbe *et al*., 2007;
259 Covelli *et al*., 2013). Figure 2 shows that lateral and polar flagellins are classified as
260 different branches, but some other flagellins belong to another branch. Interestingly,
261 branches for the lateral and polar flagellins comprise flagellins from
262 gamma-proteobacteria. The other bacteria, i.e. *Azospirillum brasilense*, *R. sphaeroides*
263 and *B. japonicum*, are classified as alpha-proteobacteria.
264 Among the strain A1 flagellins, p5 and p5′ were not categorised in the branch
265 for lateral or polar flagellin. On the other hand, p6 was positioned at the lateral flagellin
266 branch of gamma-proteobacteria (Fig. 2), although sphingomonads are classified as
267 alpha-proteobacteria. These results are consistent with a previous analysis, which
268 showed that p6 was most homologous to the lateral flagellin from *V. parahaemolyticus*
269 (Hashimoto *et al*., 2005). Data about flagellation and motility of other sphingomonads is
270 limited. Both of motile and non-motile strains have been reported (Miake *et al*., 1995;
271 Yabuuchi *et al*., 2001) and the motile strains express a single polar flagellum.
272 Genome-sequenced sphingomonads, *Sphingomonas wittichii* and *Sphingomonas* sp.
273 strain MM-1, have flagellar genes with two (*swit_1283* and *1284*) and five (*G432_0590*,
274 *06930*, *06935*, *06940* and *06940*) flagellins, respectively. These flagellins were present
275 at the branches of alpha-proteobacteria flagellins, but not at those between p6 and the
276 lateral flagellins of gamma-proteobacteria. These observations suggest that the p6 gene
277 was acquired via horizontal gene transfer after strain A1 diverged from other
278 sphingomonads.

Similarities with the lateral flagellar genes were also detected for other genes in flagellar gene cluster set I. For example, BLAST searches against the UniProtKB and SwissProt databases using the nucleotide sequences of *sph1800* (*motB*), *sph1801* (*motA*) and *sph1802* (*fliA*) as query sequences showed that they shared high homology with *lafU*, *lafT* and *lafS* from *V. haemolyticus*, respectively.

The position in the flagellin phylogenetic tree, as well as the arrangement of the set I flagellar genes mentioned above, suggests that p6 is a lateral flagellin, which is typically designated as LafA, whereas p5 and p5’ may be different from lateral flagellins based on their primary sequences.

Motility expression and flagellum formation by strain A1

The motility of cells was screened on semisolid AHY plates with 0.5% agar. First, the strain A1 cells streaked onto semisolid media exhibited no movement, but the cells began to spread. The cells at the edge of a single colony grown for a week were sub-cultured to a fresh semisolid AHY plate. In contrast to the wild-type strain A1 cells, sub-cultured strain A1 cells spread over the medium within a few days (Fig. 3(a)). We selected cells (designated as A1-M5) from the colony with the widest spread and used these cells in our further analyses. Western blotting analysis using anti-p5 antisera showed that A1-M5 cells produced a large amount of p5 compared with the wild-type strain A1 cells (Fig. 3(b)). The cross-reacted protein bands in A1-M5 cell extracts (Fig. 3(b) lanes 3 and 4) indicated the presence of other flagellins: p5′ and p6. Both the wild-type and motile strain A1 cells grew well on alginate (Fig. 3(c)). A1-M5 cells were also confirmed to exhibit a swimming ability by light microscopy (Movie S1 and S2)

and this was supported by the cell movement on 0.3% agar plate (Fig. 3(d)). TEM analysis showed that about half of A1-M5 cells formed a single mono-polar flagellum (Fig. 4), which has not been observed previously. The length and thickness of the flagellar filament were determined as ca 8 μm and 20 nm, respectively. Motility expression of strain A1 was confirmed to be reproducible. Furthermore, although initial experiment for the motility screening was undergone using semisolid AHY plates and cultivation in AHY liquid media increased the p5 expression in wild-type strain A1 (Fig. 3(b), lane 2), streaking of wild-type strain A1 onto semisolid ALY plates also induced cell motility, suggesting that high concentration of yeast extract is effective but not necessary on flagellation of strain A1.

As described above, strain A1 possesses three flagellin genes and two sets of flagellar genes. In order to determine the genes involved in the formation of the single polar flagellum in A1-M5, the gene expression levels were compared in wild-type and A1-M5 cells based on DNA microarray analyses. As shown in Table 2, all of the flagellar genes in the A1-M5 cells were expressed at much higher levels than those in the wild-type cells. The flagellar gene sets did not exhibit any obvious tendencies to be expressed preferentially. The expression levels of most of the chemotaxis-related genes also increased, but not all. The most dramatic increase (404-fold) was observed in *sph821*, which encodes a methyl-accepting chemotaxis protein that act as a receptor in chemotaxis. The expression levels of three flagellin genes, i.e. p5, p5′ and p6, increased 67-, 57- and 93-fold, respectively, in A1-M5 cells, thereby indicating that all of them were transcribed inductively in A1-M5 cells. However, the transcription levels of these flagellins were different (p5, 39242; p5′, 6054; p6, 44317). The transcriptions of *flhD*

and *flhC* genes whose products promote expression of the other flagellar genes in *Salmonella* and their related species were also highly upregulated in the A1-M5 cells. However, because the common motif for FlhD2C2 binding was not found in the strain A1 genome, the regulation of expression of flagellar genes remains to be clarified.

Because flagellar formation of the A1-M5 cells are stable, some kinds of genetic changes are supposed to occur. The similar phenomenon that flagellar formation in non-motile *Actionobacillus pleuropneumoniae* was facilitated in the soft agar media has previously been reported in the paper of Negrete-Abascal *et al.*, 2003. There is also a possibility that the strain A1 cell produces lateral flagella under the appropriate condition as reported in the case of second flagellar system of *R. sphaeroides* (Poggio *et al*., 2007). However, we failed to find such condition to date and strain A1 cells producing lateral flagella

Flagellar filaments with two distinct types of flagellins

To investigate the flagellar constituents, the flagellar filaments were sheared off from A1-M5 cells and subjected to SDS-PAGE. Two major protein bands were observed with molecular sizes of 39 kDa and 30 kDa (Fig. 5). The N-terminal amino acid sequence of the 39-kDa protein was determined as NH_2-AMTINTN, which corresponded to 2–8 amino acid residues in p5 and p5′. However, we failed to determine the N-terminal sequence of the 30-kDa protein. The flagellar fraction was analysed further by western blotting using anti-p5 antisera. In addition to the major protein bands at 39 kDa and 30 kDa, a faint protein of 37 kDa was observed after staining with Coomassie Brilliant Blue R-250 (CBB) (Fig. 5(a)). The 37-kDa band was also detected by anti-p5 antisera

(Fig. 5(b)). Gene disruption analysis indicated that *p5* and *p6* gene-disruptants lacked the 39-kDa and 30-kDa proteins, respectively, in the flagellar fraction (Fig. 5(b) and (c)). These results demonstrate that the A1-M5 flagellar filaments mainly comprise p5 (theoretical molecular mass, 39753 Da) and p6 (31068 Da), while p5′ (39542 Da) is a minor component. These findings are consistent with the results obtained in the DNA microarray analysis.

It is known that many bacteria possess multiple genes for flagellins in their genomes (Faulds-Pain *et al*., 2011). These multiple flagellins with high shared sequence homology were probably derived via gene duplication and they often form a flagellum. For example, six flagellins are present in different positions in the flagellar filament of *Caulobacter crescentus* (Driks *et al*., 1989). By contrast, the flagellins from different flagellar gene sets share lower sequence identities and they form different types of flagella, typically polar and lateral flagella (McCater, 2004). *V. parahaemolyticus* possesses seven flagellin genes, where six flagellins from two gene clusters in one chromosome form a single polar flagellum and one flagellin in the other chromosome forms lateral flagella. The six polar flagellins in *V. parahaemolyticus* are highly homologous in terms of their size (374–384 residues) and amino acid sequence (66–77%). By contrast, the lateral flagellin shares lower sequence identity with the polar flagellins and it contains fewer amino acids (284 residues).

The A1-M5 cells produced only a single polar flagellum, although they possessed two types (lateral and polar) of flagellar gene sets. In A1-M5 cells, the characteristic polar flagellar filaments comprised homologous flagellins (p5 and p5′) as well as a lateral flagellin (p6). To the best of our knowledge, this is the first report of a

lateral flagellin forming a polar flagellum. At present, the relationships among flagella formation, the transcription of flagellin genes and the spatial arrangement of p5, p5′ and p6 in the strain A1 flagellar filament are being analysed by characterising flagellin(s)-deficient cells.

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503 **Figure legends**

504 Fig. 1. Flagellar genes in strain A1. (a) Large cluster designated as set I. (b) Smaller
505 clusters designated as set II. The gene annotations of the flagellar genes and the
506 sequence ID in the genome (*sphXXXX*) are also indicated. The genes related to
507 chemotaxis are also shown for those that clustered with flagellar genes. Grey arrows
508 indicate genes that are not related to flagella.

509

510 Fig. 2. Molecular phylogenetic tree of flagellins in bacteria with dual flagellar systems
511 and genome-sequenced sphingomonads. Abra, *A. brasilense*; Ahyd, *A. hydrophila*;
512 Bjap, *B. japonicum*; Ecol, *E. coli*; Ppro, *P. profundum*; Rcen, *R. centenum*; Rsp, *R.*
513 *sphaeroides*; laf, lateral flagellin; fla, flagellin-forming non-lateral flagella such as
514 polar, sub-polar and peritrichous flagella. p5, p5', p6, Swit_#### and G432_####
515 are flagellins from sphingomonads. Grey-shaded branches and grey-dotted branches
516 indicate the groups of lateral and polar flagellins from gamma-proteobacteria,
517 respectively.

518

519 Fig. 3. Comparison of wild-type and motile strain A1 cells. (a) Swarming assay of
520 wild-type strain A1 (left) and A1-M5 cells (right). Cells were inoculated onto
521 semisolid AHY plates containing 0.5% agar and incubated at 30°C for 3 days. (b)
522 Western blot analysis of bacterial cells (5×10^6 cells) using anti-p5 antisera. Lane 1,
523 wild-type strain A1 cells grown in ALY medium; lane 2, wild-type strain A1 cells
524 grown in AHY medium; lane 3, A1-M5 cells grown in ALY medium; lane 4, A1-M5
525 cells grown in AHY medium. (c) Growth curve of wild-type strain A1 cells (solid

line) and A1-M5 cells (dashed line) in AHY medium. The experiments were repeated three times. (d) Swimming assay of wild-type strain A1 (left) and A1-M5 cells (right). Cells were inoculated onto alginate plate (ALY medium without yeast extract) containing 0.3% agar and incubated at 30°C for 3 days.

Fig. 4. Transmission electron micrographs of A1-M5.

Fig. 5. Filament constitution of the A1-M5 flagella. (a) CBB-stained image after SDS-PAGE using the flagellar filament fraction from A1-M5 cells. (b) Western blot analysis of the flagellar filament fraction using anti-p5 antisera. Lane 1, wild-type strain A1; lane 2, A1-M5; lane 3, *p5* gene-disruptant; lane 4, *p6* gene-disruptant. (c) Western blot analysis of the flagellar filament fraction using anti-p6 antisera. Lane 1, wild-type strain A1; lane 2, A1-M5; lane 3, *p5* gene-disruptant; lane 4, *p6* gene-disruptant. In each lane of (b) and (c), the flagellar filament prepared from 200 μL of bacterial cell culture (OD=1.0) was loaded.

Movie S1. Light microscopy of wild-type strain A1.

Movie S2. Light microscopy of A1-M5.

546

547 Table 1. σ28 recognition sequences at upstream of flagellar genes.

gene ID	Sequences
sph223 (*p5*)	**TTCAAGTTT**caaagcgccgg**GCCGATG**
sph242 (*motA*)	**TTCAAGTTC**gaacgaggtac**GCCGTTA**
sph246 (*cheY*)	**CTAAAGGCC**tacgccgtcgc**GCCGATA**
sph539 (*flgG*)	**TTAAAGATT**tgccttggccg**GCCGTAA**
sph1057 (*flgM*)	**CTAAAGTTT**tgtatcggtgg**GCCGTTA**

548

549

Table 2. Transcription of genes involved in flagellar formation and chemotaxis in wild-type (WT) and motile (M5) strain A1 cells.

gene ID	transcription level		change (fold)	p-value	annotation
	WT	M5			
sph183	76	581	7.7	5.0E-04	*flgL*
sph184	62	846	13.7	6.1E-06	*flgK*
sph223	581	39242	67.5	9.5E-08	*p5*
sph224	106	6054	57.3	2.2E-06	*p5'*
sph241	191	3387	17.8	1.4E-06	*motB*
sph242	240	6117	25.5	1.3E-05	*motA*
sph243	477	9410	19.7	5.6E-07	*flhC*
sph244	425	8446	19.9	1.1E-07	*flhD*
sph245	50	1921	38	2.8E-03	*cheY*
sph539	681	9367	13.8	1.3E-08	*flaG*
sph540	484	15956	32.9	3.1E-07	*fliD*
sph541	563	21240	37.7	1.5E-10	*fliS*
sph542	394	14235	36.2	1.1E-06	*fliT*
sph543	40	1197	29.9	1.3E-08	*fliK*
sph544	175	3504	20	7.8E-09	*fhlB*
sph821	38	15189	404.2	1.1E-06	*mcp*
sph1045	36	452	12.5	2.1E-03	*flgJ*
sph1046	72	1211	16.7	4.1E-04	*flgI*
sph1047	676	10850	16.1	1.2E-07	*flgH*
sph1048	389	7686	19.8	5.9E-07	*flgG*
sph1049	113	2899	25.6	2.6E-08	*flgF*
sph1050	351	7242	20.7	2.0E-07	*flgE*
sph1051	443	9126	20.6	2.9E-08	*flgD*
sph1052	1268	18799	14.8	1.1E-07	*flgC*
sph1053	354	2919	8.2	6.3E-08	*flgB*
sph1054	154	857	5.5	2.3E-04	*flgA*
sph1057	117	524	4.5	6.9E-07	*flgM*
sph1058	444	1977	4.5	4.1E-08	*flgN*
sph1225	74	8567	116.4	3.3E-05	*mcp*
sph1226	116	20446	176.5	6.3E-09	*cheY*
sph1228	53	7539	143.5	2.8E-06	*cheA*
sph1229	43	6462	151.1	2.9E-06	*mcp*
sph1230	36	2244	62.7	5.8E-05	*cheW*
sph1231	55	3297	60.5	4.8E-06	*cheR*
sph1232	50	1419	28.7	1.2E-07	*cheB*
sph1784	202	7494	37.1	1.7E-08	*flgL*
sph1786	141	5366	38.1	3.9E-09	*flgK*
sph1787	191	5083	26.6	3.2E-12	*flgJ*

sph1788	68	1694	25	6.2E-06	*flgI*
sph1789	56	1565	28	2.6E-03	*flgH*
sph1790	239	6992	29.2	3.5E-06	*flgG*
sph1791	230	8279	36	1.5E-06	*flgF*
sph1792	178	8844	49.6	2.5E-06	*flgE*
sph1793	119	8486	71.5	2.4E-07	*flgD*
sph1794	203	10113	49.8	2.2E-08	*flgC*
sph1795	408	4009	9.8	9.1E-06	*flgB*
sph1796	447	3130	7	1.5E-07	*flgA*
sph1797	36	211	5.8	1.5E-06	*flgJ*
sph1798	25	156	6.2	2.8E-04	*flgN*
sph1800	38	987	25.8	4.6E-02	*lafU*(*motB*)
sph1801	29	1240	42.2	9.1E-07	*lafT*(*motA*)
sph1802	22	539	24.2	2.4E-05	*lafS*(*fliA*)
sph1803	75	4913	65.3	1.8E-06	*lafF*(*fliL*)
sph1804	32	1947	60.3	1.4E-05	*fliK*
sph1807	26	3723	144.6	7.0E-09	*fliS*
sph1808	52	10549	202.7	4.1E-06	*fliD*
sph1809	28	1119	39.6	5.1E-08	*fliJ*
sph1810	35	487	14.1	7.0E-01	*fliI*
sph1811	28	341	12.1	2.1E-05	*fliH*
sph1812	34	572	16.6	1.6E-04	*fliG*
sph1813	38	531	14	5.5E-04	*fliF*
sph1814	87	2563	29.4	3.4E-10	*fliE*
sph1816	27	408	15.2	3.0E-04	*fliM*
sph1817	37	1149	30.8	5.1E-07	*fliN*
sph1818	57	595	10.4	1.4E-08	*fliP*
sph1819	40	150	3.7	2.8E-04	*fliQ*
sph1820	31	172	5.5	1.1E-05	*fliR*
sph1821	39	255	6.6	7.5E-01	*flhB*
sph1822	28	301	10.8	7.9E-01	*flhA*
sph1824	475	44317	93.2	3.2E-08	*p6*
sph2312	31	3010	95.6	1.7E-07	*mcp*
sph2313	78	5868	75.4	3.5E-04	*mcp*
sph2504	255	2059	8.1	1.2E-06	*mcp*
sph2859	41	1557	37.6	7.3E-08	*mcp*
sph2942	168	5861	34.9	8.9E-06	*mcp*
sph3649	62	4762	77.1	5.1E-08	*cheR*
sph3658	179	9875	55.1	9.7E-05	*cheW*
sph3659	529	32640	61.7	1.2E-09	*cheW*
sph3660	366	32698	89.3	8.5E-12	*cheY*
sph3661	193	25684	133	1.1E-07	*cheZ*
sph3662	99	8030	81.3	1.1E-05	*cheA*
sph3741	101	8119	80.7	2.6E-06	*mcp*

sph3884	46	563	12.3	2.0E-04	*flhB*
sph3885	163	2155	13.2	1.0E-06	*flhA*
sph3886	58	841	14.6	8.5E-03	*flhF*
sph3887	128	1943	14.5	1.8E-06	*flhG*
sph3888	134	1943	14.5	2.0E-08	*fliA*
sph3889	137	1534	11.2	4.9E-10	*motA*
sph3931	178	303	1.7	2.3E-01	*fliR*
sph3932	46	199	4.4	8.4E-07	*fliQ*
sph3933	129	377	2.9	6.2E-05	*fliP*
sph3939	152	5072	33.3	2.4E-09	*fliN*
sph3940	161	4283	26.6	5.8E-04	*fliM*
sph3941	253	5033	19.9	4.9E-07	*fliL*
sph3942	419	1822	4.4	1.8E-04	*fliK*
sph3944	213	1347	6.3	3.0E-10	*fliJ*
sph3945	48	670	14	5.7E-05	*fliI*
sph3946	31	549	17.6	2.9E-07	*fliH*
sph3947	70	1062	15.3	3.4E-08	*fliG*
sph3948	56	710	12.6	1.1E-06	*fliF*
sph3951	165	2331	14.2	2.0E-08	*fliE*

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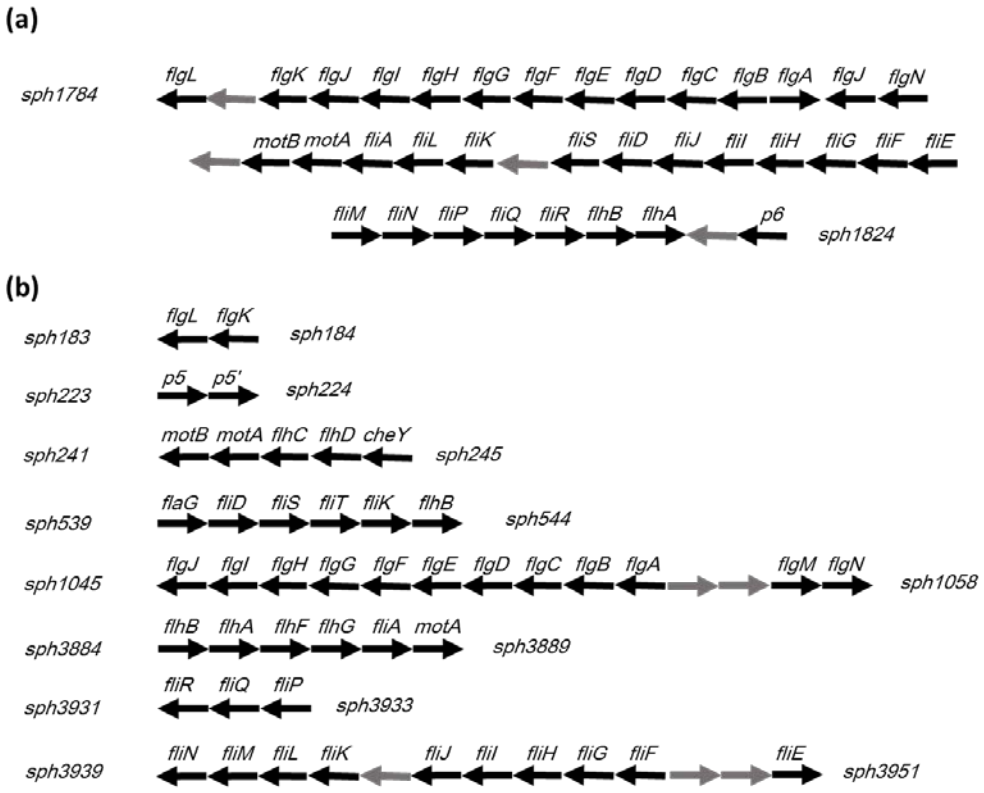

Fig. 1

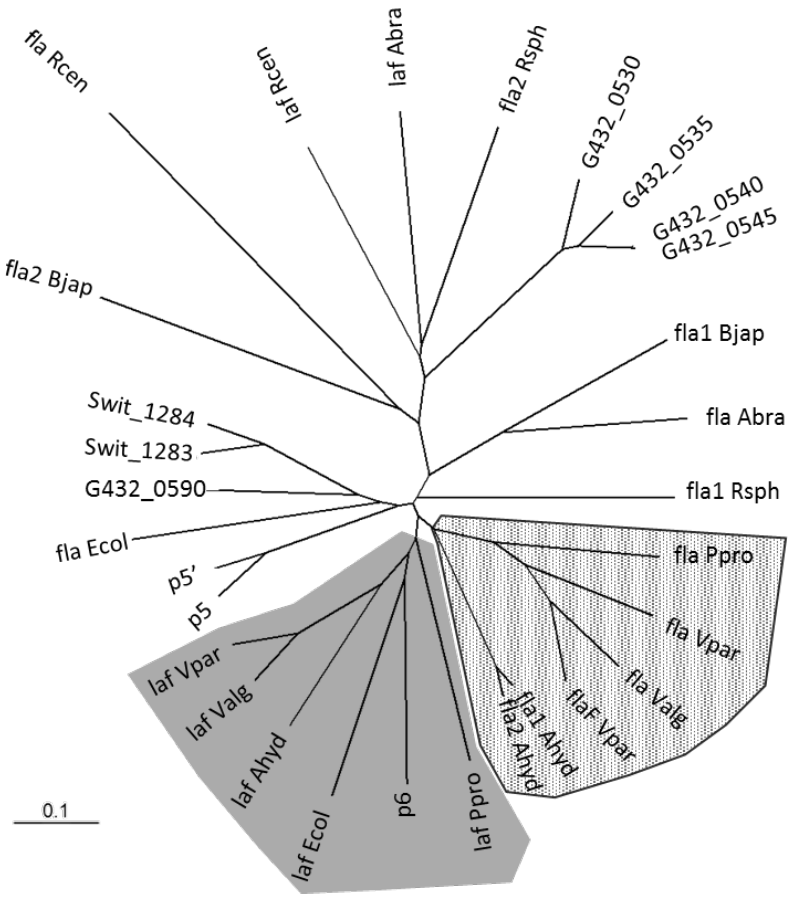

Fig. 2

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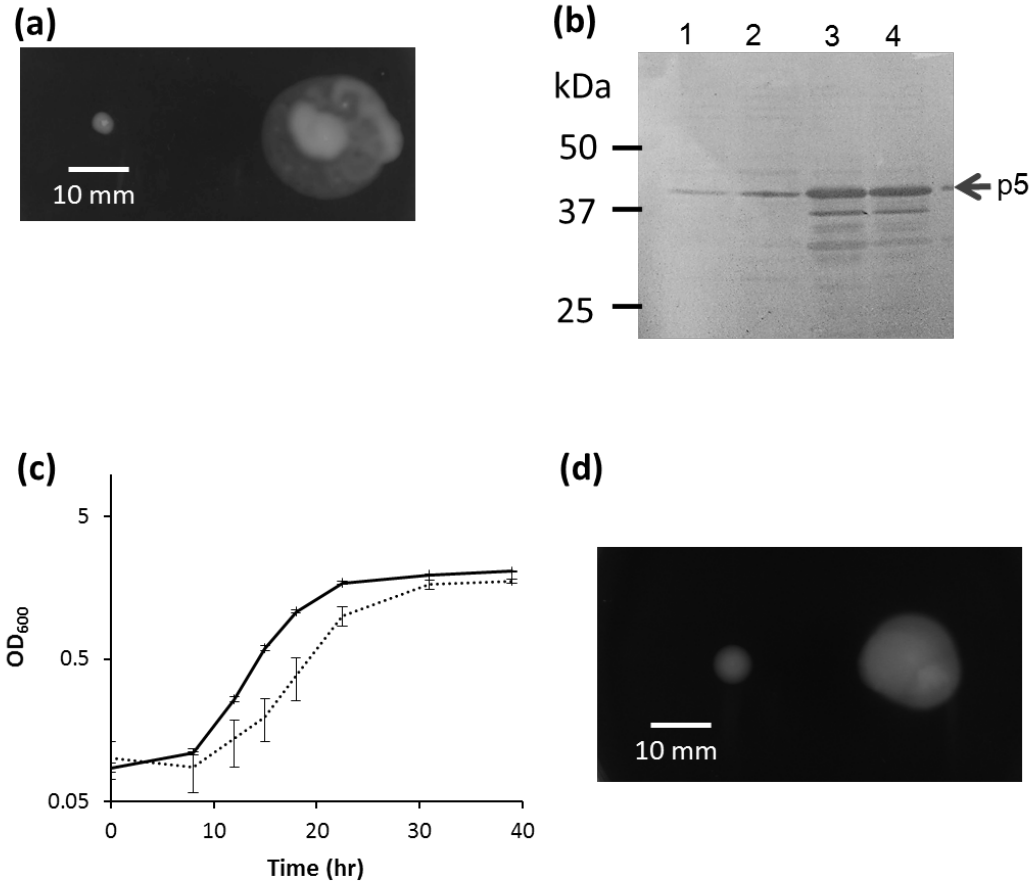

Fig. 3

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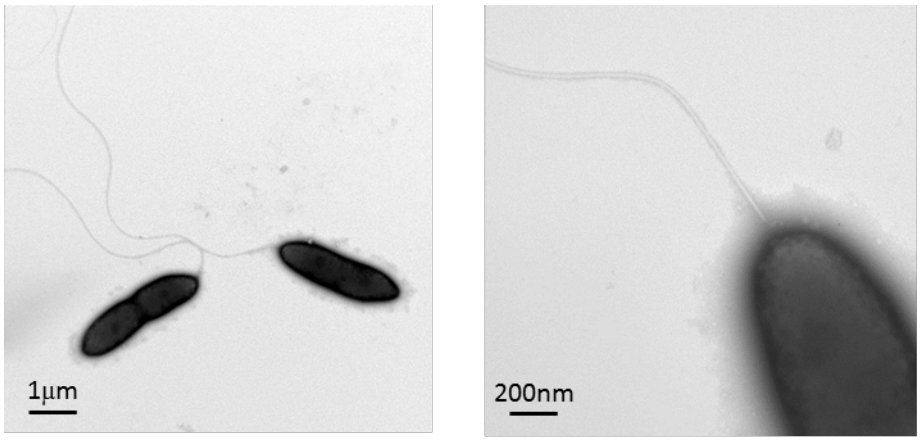

Fig. 4

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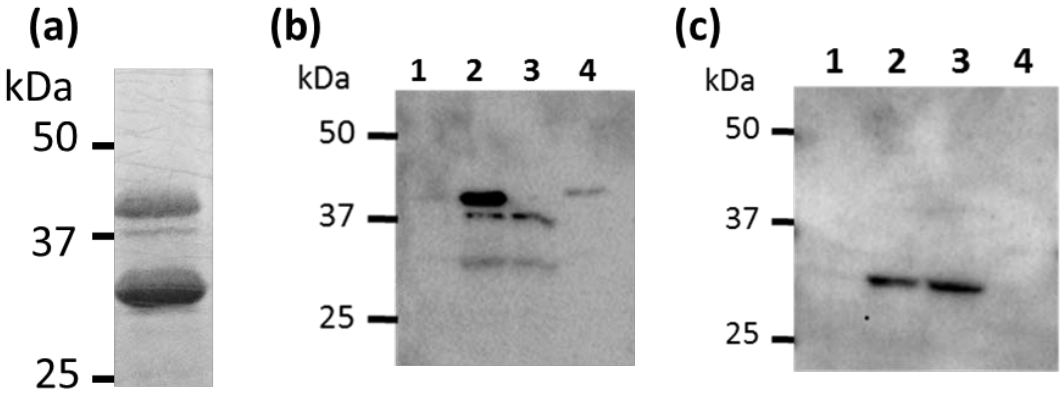

Fig. 5

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